

## Storage of ram semen at 5 °C: effects of preservation period and timed artificial insemination on pregnancy rate in ewes

A. Menchaca<sup>1,2,3</sup>, A. Pinczak<sup>2</sup>, D. Queirolo<sup>2</sup>

<sup>1</sup>Departamento de Fisiología, Facultad de Veterinaria, Lasplacas 1550, Montevideo, Uruguay.

<sup>2</sup>Gensur Ltd., Nueva York 1690, Montevideo, Uruguay.

### Abstract

Artificial insemination (AI) using chilled ram semen has not been adopted in Uruguay yet, probably due to a lack of information about the use of this technology in the country. This study evaluated the fertility rate obtained in Merino ewes inseminated with ram semen stored at 5 °C in a TRIS-based extender in Uruguayan field conditions. The effect of storage period of semen (Experiment 1) and different times of artificial insemination (Experiment 2) were studied. In Experiment 1, fresh semen was diluted in a TRIS-based extender to a concentration of 200 x 10<sup>6</sup> spermatozoa/0.2 ml. Semen, maintained at low temperature, was used for cervical insemination at spontaneous estrus, soon after collection (n = 87), or 12 (n = 75) or 24 hours (n = 84) after collection. The cooling-rate curve was adjusted at 0.25 °C/minute, and semen was cooled until it reached a temperature of 5 °C. In Experiment 2, semen was processed in a similar fashion to that of Experiment 1. Estrus was synchronized using a Short-Term Protocol using a progestagen treatment (6 days) with injection of a prostaglandin F2 alpha analogue at sponge insertion and equine chorionic gonadotropin (250 IU) at sponge removal. Insemination was performed 12 hours after estrus detection (n = 49) or at a fixed time (TAI) of 48 (n = 49), 54 (n = 47), or 48 and 54 hours (n = 47) after sponge withdrawal. Conception rate (pregnant ewes/inseminated ewes) and pregnancy rate (pregnant ewes/treated ewes) were evaluated 35-40 days after insemination using transrectal ultrasonography. In Experiment 1, conception rate was lower (34.5%; P < 0.05) for the 24-hour semen storage group or was similar (42.7%; P > 0.05) for the 12-hour storage group when compared to the control group (50.4%). In Experiment 2, a single TAI at 48 hours after sponge withdrawal resulted in a pregnancy rate similar to AI performed after detection of estrus (34.7% versus 34.7%, respectively). The TAI at 54 hours resulted in the lowest (10.6%; P < 0.05) pregnancy rate and the double TAI (48 and 54 hours) was intermediate (23.4%). Overall results showed that an acceptable conception rate was achieved when AI was performed after detection of estrus using semen stored at 5 °C for 12 hours in a TRIS-based extender. In addition, it is possible to avoid estrus detection by using a single TAI 48 hours after a Short-Term Protocol without affecting pregnancy rate.

**Keywords:** sheep, chilled semen, TRIS, Merino ewes.

### Introduction

Cervical artificial insemination (AI) using fresh ram semen has been widely used around the world (Gordon, 1983; Evans and Maxwell, 1987). When frozen ram semen is used, a spatial and temporal relationship exists between the time of collection and insemination. Due to low pregnancy rates resulting from cervical insemination using frozen semen, intrauterine deposition of frozen semen remains the most popular method (Salamon and Maxwell, 1995; Gil, 2001). Another alternative is the use of liquid semen stored between 0-5 °C, allowing the use of semen for a longer period of time compared to fresh semen. The use of cooled semen has resulted in higher pregnancy rates when deposited at the cervix compared with frozen semen (reviewed by Maxwell and Salamon, 1993; Salamon and Maxwell, 2000). Different diluents for chilled semen have been used to improve fertility rate. A TRIS-based extender was recommended by Evans and Maxwell (1987). However, there are few studies that have used liquid semen stored for different periods of time in TRIS-based extender in field conditions in Uruguay (Gil and Olivera, unpublished data). This technology could be considered a useful tool in countries such as Uruguay due to short distances between farms, adequate road connections, existence of ram reproductive centers, and widespread utilization of AI in sheep.

Timed artificial insemination (TAI) seems to be advantageous when compared to AI programs using estrus detection. The use of a Short-Term Protocol with a progestagen treatment (5-7 days) before TAI using fresh semen in ewes and goats has been successful (Menchaca and Rubianes, 2004). Time of ovulation is closely synchronized with ovulation occurring on average 60 hours after the end of a Short-Term Protocol. Acceptable pregnancy rates were obtained when this method was coupled with TAI in goats (Rubianes and Menchaca, 2003). The optimal time of TAI after a Short-Term Protocol using liquid semen stored at 5 °C has not been evaluated in ewes yet.

In this study, we evaluated the pregnancy rate obtained using semen stored at 5 °C in a TRIS-based extender after different durations of storage. We also determined the optimal time for TAI using chilled semen after a Short-Term Protocol in ewes managed in field conditions in Uruguay.

<sup>3</sup>Corresponding author: alejomen@adinet.com.uy

Received: April 3, 2005

Accepted: February 13, 2006



## Materials and Methods

### *Animals and management*

The study was performed at a Merino sheep farm (Rio Negro, Uruguay, 33° SL) during the breeding season (February). The ewes were kept as a single flock in an extensive productive system stocked at 1-2 animals/hectare grazing on native pastures of typical rangelands in Uruguay. Two experiments were conducted in field conditions using a total of 438 multiparous Merino ewes with body condition scores (BCS) of 2.0 to 3.5 (scale 0 to 5). Ewes were assigned to experimental treatment groups in a fashion so that BCS was equalized between groups.

### *Semen processing*

Semen was collected using an artificial vagina from three mature Merino Dohne rams maintained on a semen collection regimen that started 15 days before the experiments began. One or two (pooled per ram) consecutive ejaculates from each ram were used every day. All ejaculates were evaluated using light microscopy within 10 minutes after collection. Ejaculates were only used if the volume was  $\geq 0.75$  ml and had a sperm concentration  $\geq 2.5 \times 10^9$  spermatozoa/ml and  $\geq 70\%$  motile cells. After measuring the sperm concentration (Neubauer chamber, 100x magnification), semen was diluted at final concentration of  $1 \times 10^7$  spermatozoa/ml. Each dose was calculated to yield  $200 \times 10^6$  spermatozoa in a volume of 0.2 ml. Semen was extended using a TRIS-based extender (Salamon and Maxwell, 2000) in a solution of double-distilled water containing TRIS (3.63 g), glucose (0.5 g), citric acid (1.99 g), and supplemented with 15% (v/v) egg yolk. Penicillin and streptomycin were added to the extender (100,000 IU and 100 mg, respectively). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Semen was maintained in a water bath at 30 °C for 15-20 minutes until dilution. After that, subjective motility was re-evaluated, and only semen having at least 70% of motile cells was used. The temperature and cooling-rate curve was adjusted to 0.25 °C/minute and semen was cooled until it reached 5°C.

Semen from different rams was equally distributed over the different groups in each experiment. Semen was placed at the external cervical os with the aid of a tubular vaginoscope equipped with a light source and a multidose insemination gun (Walmur-Veterinary Instruments, Montevideo, Uruguay).

### *Experiment 1*

Artificial insemination (AI) of 246 ewes was performed 10 to 14 hours after spontaneous estrus.

Ewes were randomly inseminated with TRIS-based extended semen without refrigeration (at 30°C) as control group (group 0 h, n = 87) or after storage at 5°C for 12 (group 12 h, n = 75) or 24 hours (group 24 h, n = 84) after collection. Estrus was synchronized 15 days before the experiment, and AI was performed after the next spontaneous estrus. Estrus behavior was checked for twice daily for 5 days using androgenized, painted wethers. Teaser wethers were treated with three doses of testosterone ciclopentilpropionate (i.m., 200 mg per dose, Dispert, Uruguay), given at 15, 7, and 1 day before the beginning of the estrus detection period. Teasers were painted twice daily and remained with the ewes in a 1:10 ratio.

### *Experiment 2*

Estrus was synchronized in 192 ewes using a Short-Term Protocol (Menchaca and Rubianes, 2004). The protocol was performed using intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Syntex, Buenos Aires, Argentina) in conjunction with an intramuscular dose of a prostaglandin F2 alpha analogue (160 µg delprostenate, Glandinex, Universal Lab, Uruguay) at sponge insertion. Equine chorionic gonadotropin (250 IU, Novormon, Syntex, Argentina) was administered 6 days later at sponge withdrawal. In the estrus-detected group (group ED; n = 49), estrus was checked twice daily for 72 hours after sponge withdrawal, and AI was performed 12 h after estrus detection. The other groups received a single TAI at 48 (group TAI-48 h; n = 49), 54 (group TAI-54 h; n = 47), or 48 and 54 hours (group TAI- 48 and 54 h; n = 47) after sponge withdrawal. Semen was maintained at 5 °C and used 12 hours after collection. Pregnancy was determined 35-40 days after AI by transrectal ultrasonography using a 5.0 MHz linear probe (Aloka 500, Japan).

### *Statistical analysis*

Conception (pregnant ewes/inseminated ewes) and pregnancy (pregnant ewes/treated ewes) rates were analyzed in each experiment using logistic regression (Stata, 2003) including the effect of duration of semen storage (Experiment 1) or timing of TAI (Experiment 2). The analysis also included the effect of the ram, BCS of the ewes, and interactions with treatment effects. Differences were considered significant at  $\alpha = 0.05$ .

## Results

The conception rate obtained in Experiment 1 using fresh semen (group 0h) was 54.0% (47/87) and did not differ from that of ewes inseminated with semen stored for 12 hours (42.7%; 32/75). The conception rate resulting from insemination with semen stored for 24 hours was lower (34.5%; 29/84) than with fresh semen ( $P < 0.05$ ). In the ED group of Experiment 2, estrus was



detected in 81.6% (40/49) of the ewes. Similar to Experiment 1, the conception rate in these ED ewes inseminated with semen stored for 12 hours was 42.5%

(17/40). Pregnancy rates from Experiment 2 are presented in Table 1. The results for both experiments were not affected by the ram or the BCS of the ewes.

Table 1. Pregnancy rates obtained using ram semen stored for 12 hours at 5 °C with artificial insemination (AI) performed 12 hours after estrus detection or timed AI (TAI) at 48, 54, or 48 and 54 hours after sponge withdrawal.

	AI after estrus detection	TAI 48 h	TAI 54 h	TAI 48 and 54 h
Pregnancy rate (%)	34.7%	34.7%	10.6%	23.4%
(n)	(17/49) <sup>a</sup>	(17/49) <sup>a</sup>	(5/47) <sup>b</sup>	(11/47) <sup>ab</sup>

<sup>ab</sup>Different letters indicate significant differences, ( $P < 0.05$ ) between treatments (Experiment 2).

### Discussion

The present study shows that acceptable pregnancy rates can be obtained using liquid ram semen stored for 12 hours at 5 °C that is deposited at the cervix via a single TAI 48 hours after sponge withdrawal. In Experiment 1, the conception rate following AI after spontaneous estrus using fresh semen was 54.0%. This result did not differ significantly from the conception rate obtained with AI using semen stored for 12 hours (42.7%). Storage of semen for 24 hours severely affected the conception rate with a 20 % lower fertility rate than that obtained using fresh semen (34.5% *versus* 54.0%, respectively,  $P < 0.05$ ). In a study using Norwegian Crossbred ewes, Paulenz *et al.* (2003) reported a 51.5% lambing rate using semen stored at 5 °C diluted in a commercial TRIS-based extender. In the present study, the lower pregnancy rate was probably caused by different factors such as biotype and sheep management. Extensive animal management in Uruguayan field conditions has its own characteristics. In these conditions, a detrimental effect on pregnancy rate in ewes with a body condition score lower than 2.75 (scale 0 to 5) was demonstrated (Menchaca *et al.*, 2003). In the present study, we worked with the typical BCS of sheep in this country, ranging from 2.0 to 3.5. Usually, conception rate in these conditions using a single AI with fresh semen ranges from 50 to 60% (Durán del Campo, 1993). Therefore, in this experiment, we consider the pregnancy rate (42.7%) obtained using semen stored for 12 hours acceptable. In summary, Experiment 1 demonstrated that semen stored at 5° C should be used within 12 hours from collection to avoid a highly detrimental effect of storage time on the conception rate.

In Experiment 2, using semen stored at 5 °C for 12 hours, we obtained similar pregnancy rates for AI performed relative to detected estrus (34.7%) and TAI at 48 hours after the end of a short-term progestagen treatment (34.7 %). These pregnancy rates were higher than those ewes submitted to TAI at 54 hours (10.6%;  $P < 0.05$ ) and were not affected by double TAI at 48 and 54 hours (23.4%,  $P > 0.05$ ).

Probably, the higher pregnancy rate that resulted from AI at 48 hours after sponge withdrawal is likely due to the fact that AI was performed at a time closer to ovulation. The timing of ovulation following a Short-Term Protocol in small ruminants occurs around 60 hours after sponge withdrawal (Menchaca and Rubianes, 2004). However, a small dispersion in the period over which ovulation occurs in the flock could affect the pregnancy rate when cervical AI is performed close to 60 hours (i.e. 54 hours) after sponge withdrawal. The period of time of sperm transportation through the female reproductive tract (Maxwell and Salamon, 1993) should be also considered as one factor affecting pregnancy rate in ewes submitted to TAI at 54 hours. These two phenomena could explain the differences in pregnancy rates between TAI at 48 *versus* 54 hours after sponge withdrawal. In summary, this experiment shows the possibility of avoiding estrus detection and using a single TAI 48 hours after a Short-Term Protocol.

In conclusion, it is possible to obtain acceptable conception rates with artificially-inseminated ewes after estrus detection using liquid ram semen stored at 5 °C for 12 hours, when compared to AI using fresh semen. In addition, a single TAI at 48 hours after the end of Short-Term Protocol using semen stored for 12 hours resulted in a similar pregnancy rate to that of AI after estrus detection. Therefore, these results showed that the storage of ram semen for 12 hours and its deposition at the cervix via a single TAI without estrus detection results in acceptable pregnancy rates. Both of these aspects have important benefits to AI programs applied in Uruguayan field conditions.

### Acknowledgments

We are grateful to the proprietary and the field team of the “Tres Arboles” farm for allowing the use of their facilities during the experiments, to Edgardo Rubianes for his cooperation dealing with different technical aspects of equipment (PEDECIBA, UdelaR), and Rodolfo Ungerfeld for his comments on manuscript preparation. This research was financially supported by Merino Dohne Project of “Tres Arboles” farm.



### References

- Durán del Campo A.** 1993. *Handbook of reproduction and artificial insemination in sheep* (in Spanish). Montevideo: Hemisferio Sur. pp.165-174.
- Evans G, Maxwell C.** 1987. *Salamon's artificial insemination of sheep and goats*. Sidney: Butterworth. pp.66-76, 158-161.
- Gil Laureiro J.** 2001. *Fertility of frozen-thawed ram semen: with special reference to influence of extenders and freezing procedures*. Uppsala, Sweden: Swedish University of Agricultural Sciences. Thesis.
- Gordon I.** 1983. Fixed-time sheep artificial insemination. *In: Controlled breeding in farm animals*. Oxford, UK: Pergamon. pp.197-208.
- Maxwell WMC, Salamon S.** 1993. Liquid storage of ram semen: a review. *Reprod Fertil Dev*, 5:613-638.
- Menchaca A, Gil J, Olivera J, Rubianes J.** 2003. Effect of body condition score at time of service on fertility and fecundity in artificially inseminated ewes (in Spanish). *In: Abstracts of the V International Symposium on Animal Reproduction*, 2003, Córdoba, Argentina. Córdoba, Argentina: The Symposium. pp.404.
- Menchaca A, Rubianes E.** 2004. New treatments associated with timed artificial insemination in small ruminants. *Reprod Fertil Dev*, 16:403-414.
- Paulenz H, Söderquist L, Ådnøy T, Fossen OH, Andersen Berg K.** 2003. Effect of milk- and TRIS-based extenders on the fertility of sheep inseminated vaginally once or twice with liquid ram semen. *Theriogenology*, 60:759-766.
- Rubianes E, Menchaca A.** 2003. The pattern and manipulation of ovarian follicular growth in goats. *Anim Reprod Sci*, 78:271-287.
- Salamon S, Maxwell WMC.** 1995. Frozen storage of ram semen. II. Causes of low fertility after cervical insemination and methods of improvement. *Anim Reprod Sci*, 38:1-36.
- Salamon S, Maxwell WMC.** 2000. Storage of ram semen. *Anim Reprod Sci*, 62:77-111.
- Stata** statistical software. 2003. Release 8.0. College Station, TX: Stata Corporation.
-